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(57) Abstract

A new strain of sporogenous bacteria, ascribable to the species Bacillus coagulans is employed as an additive in animal feedstuffs. There are described the method for the preparation of both the cultures and the formulated feedstuff compositions in which said bacteria are used.

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A BACTERIAL STRAIN OF THE SPECIES BACILLUS COAGULANS: ITS USE AS A PROBIOTIC AGENT

The present invention relates to viable bacterial cells that can be used as probiotics. By probiotic, there is meant a feedstuff supplement consisting of living microbial cells that beneficially influences the animal host, thereby enhancing its intestine microbial equilibrium. The present invention also relates to formulated feedstuff compositions based on said cells. They are sporogenous bacteria, free of pathogenic potency. They have a high thermal stability and can be kept for a long time. They can possibly multiply under aerobic and anaerobic conditions and produce lactic acid and vitamin factors, as well as substances having an antagonistic selective activity to other microorganisms. The invention also relates to the use of said bacteria as single or associated cultures in the zootechnical field, in order to obtain a better physiologic-nutritional attitude of the monogastric or polygastric livestock, thereby achieving a reduction of the common pathologic breeding manifestations, lowered mortality and favourable effects on the animal growth and feedstuff conversion. The above advantages are especially relevant in the early period after the animal birth, when the risks associated with intensive breeding are usually at their highest.

It is known that by adding certain microorganisms to the animal diet, a series of advantages can be achieved, such as promoting the growth of the animal and protecting it from certain disorders. Said microorganisms act through multiple, intercrossing mechanisms, which possibly comprise the supply of certain growth, vitamin and coenzyme factors, enzyme activity as well as factors antagonizing other harmful, putrefying or pathogenic microorganisms. The mentioned mechanisms may also include the role probably played by the production of lactic acid and/or special substances of metabolic origin that have an antitoxin or antimicrobial effect, as well as the positive interferences in the functional reflexes of the digestive system and the interactions with the intestine microflora of the individual animal.

The overall result that can be obtained by using feedstuff compositions containing the above-mentioned cultures is comparable

and can be superior to that achievable with the use of certain antibiotics for stimulatory-profilactic purpose in the zootechnical field, which latter use is, however, presently not generally accepted.

In this connection various cultures of lactic bacteria belonging to the genuses Lactobacillus and Streptococcus have been tested with the aim at improving the zootechnical performance. Said cultures have a draw-back in that they are thermolabile and cannot be kept for a long time, even those which are able of reproducing "in vitro" at higher temperatures (37°C) than other cultures. The trials for increasing the cell survival times by drying these cultures, and the possibility of keeping them viable for a long time, by making use of special methods for the conservation of liophyilized preparations, i.e. by subjecting them to other physical treatments or embedding them in matrices or protecting them by means of chemicals, have not produced up to now, any satisfying results and involve anyhow remarkable additional costs.

Trials have therefore been made using microorganisms having greater resistance and better maintenance characteristics. Particularly, strains belonging to the genus Bacillus have been tested, as they have a natural thermal stability and long term maintenance characteristics and, owing to the fact that they have an endospore phase, are characterized by high resistance to heat and other chemical or physical agents. However, no significant results could be achieved on the animals they have been administered to.

It is thus an object of the present invention to provide microbial strains having the above-mentioned useful effects while being sufficiently stable and conservable in order to be possibly used on an industrial scale. What is especially needed is the resistance to a thermal treatment such as, for example, the treatment involved in an economical process for drying a cellular biomass or in the pelletization of feedstuffs; in any case the cells should be suitably preserved in whatever kind of active premix, even for many months at room temperature, as this is required for a commercially distributed

product to be added to solid, powdery, mealy or liquid concentrated feedstuff materials.

Said object has been achieved according to the present invention trough a newly isolated bacterial strain. It is a sporogenous bacterium that was isolated from ensilated fodder, i.e. an acidic environment typical of lactic bacteria. This strain has been found to be free of pathogenicity, facultatively anaerobe, thermophylic and highly heat resistant, acid tolerant and sarcolactic acid producing. It can be taxonomically assigned to the species Bacillus coagulans. A culture of said strain has been deposited under the stipulations of the Budapest Treaty on 19.3.1991 at the Institut Pasteur-Collection Nationale de Cultures de Microorganismes (C.N.C.M.), 25 rue du Docteur Roux, 75724 Paris, Cedex 15, France, under the number I-1061.

The above-mentioned strain can be cultured from a 24 hour slant to be inoculated into a 750 ml conical flask containing 100 ml of the hereinafter described culture medium, which is shaken for about 1 day and consists "in toto" of the inoculum of a prefermenter containing 2 l of the same medium. After about 18 hours of submersed culture, this second broth culture is used to inoculate a 20 liter fermenter containing a production culture medium having the following composition (in g/l of spring water): corn steep liquor 20, yeast extract 5, casein hydrolizate 2, powdered milk whey 3, monopotassium phosphate 1, spring water to 1000 ml, pH 7; sterilization at 0.75 atm for 20 minutes. The fermentation is to be carried out under the following conditions: temperature 37°C, stirrer speed 350 rpm, aeration 1 v/v/m, control of the possible foam formation by means of a silicone. The separation of the produced cellular biomass is carried out after 18-24 hours of fermentation. There is obtained a biomass yield of 5-6 g (dry basis) per liter of broth culture. The broth culture is centrifuged, washed with spring water, again centrifuged with a physiological solution and then subjected to liophyilization (approximately, 50% of spores and 50% of cells).

The lyophilized biomass is a light, straw-coloured powder containing 50-100 billions cells per gram of lyophilized product (approximately 50% in spore form) . A microbiological control of the

product kept at a temperature of 20-25°C, carried out over a period of 10 years, has shown a reduction in the number of viable cells in such a biomass in the order of 20-30% of the starting number of live cells, and has shown said reduction to be constant in time, without modification of the starting percentage of the sporification, depending on the composition of the culture medium. The product, which can be conveniently homogenized in powder form at the outlet of the liophylization device or later on at the moment of use, is mixed, by means of suitable equipment, with dextrose or corn flour in such a proportion (0.1-1%) as to provide a premix having a concentration of viable cells of 0.35-8 x 10^{11}/kg. The premix can then be added to feedstuff at a ratio resulting in a concentration of viable cells of 0.35-8 x 10^{10}/kg of final feed. This corresponds to about 100-1000 mg of lyophilized preparation/kg of diet, depending on the target animal species and the administration period. The concentration can be increased in the case of use for therapeutical purposes.

Some animal tests have provided the following results.

Example 1

There have been used 100 male broiler chicks of Ross breed divided into four groups initially comprising 25 animals each, which have been fed with one of the following formulations:

a) basal diet only; b) basal diet supplemented with 100 ppm of Elancoban (coccidiostatic agent); c) basal diet supplemented with 10 ppm of Virginiamycin (auxinic agent); d) basal diet supplented with cells of the bacterial strain of the present invention, added at a rate of 1.6 x 10^{10} CFU/kg of feedstuff for a period of 7 days and at a rate of 4.0 x 10^{9} CFU/kg of feedstuff from the seventh to the fourtyninth day. The composition of the basal diet (% by weight) was as follows: corn 55, soybean meal 30, meat meal 5, sunflower meal 5, animal fat 2.5, distillers solubles 1.5. The feedstuff was supplemented with vitamins, amino acids, sodium chloride, potassium phosphate and calcium carbonate. The feedstuff and drinking water have been administered "ad libitum" from the day 0. The animals have been kept from day 0

through day 21 in heat controlled cages at 30°C. Thereafter they have been transferred to larger cages in temperature-controlled environment with a 24 hour lightning system. The animals did not have any access to their faeces. The cages were cleaned every second day.

The following zootechnical parameters have been evaluated.

- Average weight: ratio of total group weight to the number of chicks comprised in the group (grams);

- Average daily weight increase: ratio of the weight increase of the chicks to the animal age (grams/day);

- Average daily feed consumption; ratio of the amount of feedstuff consumed per time intervall (grams/day);

- Average feed conversion ratio: ratio of feedstuff intake to weight increase. The average feed conversion ratio has been determined in the various test periods by calculating the ratio between average daily feed consumption and average daily weight increase.

At the end of the 49 day test period, divided in 5 sub-periods (0 - 7 - 21 - 35 - 49 days), the mortality due to various factors was 4% for the groups a) and d), i.e. the control group and the group feed on a diet containing cells of the bacterial strain according the present invention, respectively. It was 12% for the groups b) and c). The remaining subjects did not show any particular sign of pain in any of the test groups.

After the first week, until the end of the test period, it could be observed, with an estimation of the mean limits of confidence ($p > 0.05$), that the weight of the control group was always significantly lower than the weight of the three groups treated with food additives, the group treated with the bacterium included. At the fourtynineth (49th) day (end of the test period), compared to the average weight of the control group of 2209.4 g, the average weight and the related percent increase of these groups were: 2403.9 g and 8.8% for the group

b), treated with the coccidiostatic agent; 2373.1 g and 7.4% for the group c), treated with Virginiamycin; 2368.8 g and 7.2% for the group d) treated with the bacterium according to the invention.

As to the average feed conversion ratio, it could be found that the control group during the whole test period, had an average feed conversion ratio higher than the three treated groups, including the bacterium treated group, i.e. a lower conversion of feedstuff into live weight. Considering the three treated groups, it could be observed that the groups c) and d), fed on the diet with the added Virginiamycin and the bacterium according to the invention, respectively, had a lower average feed conversion ratio in the first three weeks, i.e. a better conversion of feedstuff into live weight as compared with the coccidiostat-treated group. It is to be noted that the animals treated with the bacterium exhibited a lower average feed conversion ratio until the end of the test. At 49 days, the average feed conversion ratio of the control group was 2.07; the average feed conversion ratio and the corresponding percent improvement relative to the control group were: 1.98 and 4.6% for the coccidiostatic agent-treated group; 2.00 and 3.5% for the Virginiamycin-treated group; 1.95 and 6.1% for the group treated with the present bacterium, which is the best result.

As a conclusion, the treatment based on the use of the bacterium of the present invention results in a growth promoting effect that can fully substitute the common practice based on sub-therapeutical doses of an antibiotic additive such as Virginiamycin, since it exhibits the same effectiveness for the weight increase and conversion of feedstuff into live weight of the animal.

Example 2

Half-breed Landrace Large White piglets have been used. Seven farrows with a total of 56 piglets have been considered (4 x 8 animals, total 32, for the group treated with a growth promoting agent, taken as the control, and 3 x 8 animal, total 24, for the piglets treated with the bacterium of the present invention), and followed from weaning (day 30) for more than 5 weeks. The treated group was fed, after weaning,

with feedstuff, medicated with only Tylosine for the prophylaxis of respiratory diseases, supplemented with 4 x 10^{10} CFU/kg of feedstuff for the first seven days and with 1 x 10^{10} CFU/kg of feedstuff for the other four weeks. The control groups were fed with the same feedstuff as used for the treated animals, but supplemented with Carbadox, a chemoprofilactic agent used as a growth promoter, instead of the bacterial biomass. The weight has been monitored at days 0 (weaning day), 27 and 35.

After weaning, the animals were reared in one room, in flat-decks, to ensure homogeneous environmental conditions.

The results, with respect to feed conversion ratio (FCR), have shown that the piglets treated with the bacterial biomass of the present invention exhibited a lower feed conversion ratio (FCR 1.76) as compared with the group treated with a commonly employed growth promoting agent (FCR 2.192). This indicates that also another livestock species the possibility of using the bacterium of the present invention as an growth promoting agent exists.

Example 3

Nine male veals, Italian Frisian breed, have been used. Three days after birth, after having been fed only the colostrum, the veals have been divided into three groups, each comprising 3 animals, and fed on one of the following formulas:

a) a commercially available feedstuff based on low fat (60%) milk, with the addition of zinc bacitracin 80 ppm (an antibiotic used as a feeding additive for growth promoting purposes);

b) a feedstuff based on low fat (60%) milk, supplemented with cells of the bacterial strain of the present invention. The amount of feedstuff and the concentration were such that the dosis of bacterial strain was 4 x 10^{10} CFU / animal / day for 4 days, until the age of seven days, and 1 x 10^{10} CFU / animal / day as from the seventh day of life. For this group also a dosis of 1.6 x 10^{11} CFU / animal / day (four times the beginning dosis) was foreseen to be administered in case of enteric

illnesses of the animals. The single daily dosis was administered suspended in rehydrated feedstuff;

c) a feedstuff based on low fat (60%) milk as it is, i.e. without any supplementation.

The animals were monitored daily for:

- behaviour (appetite, vitality, appearance of excrements);

- possible signs of the most common enteric and respiratory pathologic conditions;

- mortality.

Those animals from the groups a) and c) that became ill were treated with the usual therapeutical methods.

Group a), treated with zinc bacitracin, did not exhibit any special health problems; but for one of the three animals, three therapeutical interventions for enteritis were necessary.

Group b), treated with cells of the bacterial strain of the invention, at the beginning dosis of 4×10^{10} CFU / animal / day did not exhibit any special health problems. However, with the reduction of the dosis to 1/4 (1×10^{10} CFU / animal / day):

- one animal had a serious diarrhoea followed by an inflammation of the respiratory apparatus and died three days later, although treated with an antibiotic;

- two animals exhibited a low diarrhoea which was reduced by reducing the daily amount of milk and by adding common commercial rehydrating preparations. After 5 days of the low dosis cell administration, said animals exhibited medium diarrhoea; therefore they were treated for the next 5 days with the higher foreseen dosis (1.6×10^{10} CFU / animal / day), thereby achieving an immediate, steady improvement. Because of the general feebleness of the animals, a lung desease began, which was overcome by means of

a three-day antibiotic treatment. Thereafter, the dosis of 4×10^{10} CFU / animal / day was restored until the twentyfirst day, and then again lowered to 1×10^{10} CFU / animal / day until the end of the test period. No further antibiotic treatments were needed during this period.

Group c), devoid of any treatment, suffered right from the beginning under lack of appetite and light diarrhoea; seven days later, serious diarrhoea and fever, due to an inflammation of the respiratory apparatus, appeared, which required a therapeutic intervention, despite of which the animals died the next day.

The intervention at the maximum foreseen dosis has indicated a certain activity which can also be interpreted as a therapeutic activity attributable to the described bacterial strain.

Claims

1. A new bacterial strain of the species Bacillus coagulans, strain C.N.C.M. I-1061, a functional equivalent, subculture, mutant or variant thereof.

2. Biologically pure cultures of the bacterial strain of the species Bacillus coagulans, strain C.N.C.M. I-1061.

3. Spores of the bacterial strain according to claim 1 or 2.

4. The use of the bacterial strain of the species Bacillus coagulans, C.N.C.M. I-1061, as an additive in animal nutrition.

5. The use of the bacterial strain C.N.C.M. I-1061 in the preparation of feed supplements with probiotic, auxinic and therapeutic effects on live-stock.

6. A feedstuff premix comprising cells and spores of the bacterial strain C.N.C.M. I-1061, at a concentration which is comprised from about 10 to about 1000 millions of viable cells per gram of premix.

7. A process for the manufacture of a feedstuff premix as claimed in claim 6, comprising adding to a basic feedstuff a bacterial strain according to any one of claims 1 to 3.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/00030

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/20; A23K1/16; //(C12N1/20,C12R1:07)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12R ; A23K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	GB,A,1 204 017 (SANKYO COMPANY LIMITED) 3 September 1970 see the whole document ---	1-7
A	DE,A,1 692 490 (SANKYO CO. LTD.,) 5 August 1971 see the whole document -----	1-7

° Special categories of cited documents : [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 APRIL 1993	10. 05. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9300030
SA 68820

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-1204017	03-09-70	None	
DE-A-1692490	05-08-71	FR-A- 1502961 NL-A- 6615008	24-04-67